Constitutively Dead, Conditionally Live HIV-1 Genomes

EX VIVO IMPLICATIONS FOR A LIVE VIRUS VACCINE*

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An effective vaccine against AIDS is unlikely to be available for many years. As we approach two decades since the first identification of human immunodeficiency virus, type 1 (HIV-1), currently, only one subunit vaccine candidate has reached phase 3 of clinical trials. The subunit approach has been criticized for its inability to elicit effectively cytotoxic T-lymphocyte (CTL) response, which is felt by many to be needed for protection against HIV-1 infection. In subhuman primates, a live attenuated simian immunodeficiency virus (SIV) vaccine candidate, capable of inducing CTL, has been found to confer prophylactic immunity sufficient to prevent simian AIDS. Because replication competent (live) attenuated viruses could over time revert to virulence, such a live attenuated approach has largely been dismissed for HIV-1. Here, we describe the creation of constitutively dead conditionally live (CDCL) HIV-1 genomes. These genomes are constitutively defective for the Tat/TAR axis and are conditionally dependent on tetracycline for attenuated replication with robust expression of viral antigens. Our results suggest that CDCL genomes merit consideration as safer "live" attenuated HIV-1 vaccine candidates.

A protective vaccine against HIV-1[1] remains elusive. Intensive attempts at producing a subunit-based, particle vaccine have led to only one vaccine candidate of this type to reach phase 3 clinical trial. Indeed, it is even unclear that subunit vaccines could inherently provide sufficient protection against HIV-1 (1). In the macaque model of AIDS, subunit particles have consistently failed to induce protective immunity (reviewed in Ref. 2). By contrast, in the same macaque model, a live attenuated virus approach has worked efficaciously. Several investigators have shown that macaques inoculated with replication-competent SIV, attenuated by virtue of deletions in the viral genome, developed an immune response, which protected the animals from subsequent challenge with wild-type virus (3–6). Paradoxically the same attenuated SIV inocula, which induced protective immunity in most animals, could cause disease in a minority of other animals (5, 7, 8). Thus, these live SIV vaccine results highlight a paramount concern, which confronts the development of a live attenuated HIV-1 vaccine: the potential for vaccine-induced "breakthrough" infections.

Extant findings in humans suggest that such safety concern is more than academic. An Australian cohort infected with HIV-1 via blood product transfusion from a single donor (9) serves as an illustration. Initially, these individuals maintained stable CD4 lymphocyte counts and showed no overt clinical progression for 10–14 years (the donor gave blood over a 3.5-year period). When the infecting HIV-1 was characterized, it was found to contain deletions in U3 LTR- and *nef*-coding regions, suggesting such changes sufficiently attenuated pathogenicity potentially creating a safe vaccine virus. However, subsequent follow-up (~16 years after infection) data were instructive: CD4 lymphocyte decline and increasing HIV-1 loads did become evident in the original blood donor and two sero-converted recipients (10). Thus, this cohort testifies to a very real safety concern for live HIV vaccine: the potential evolution of an attenuated live virus to virulence.

Current state of live HIV vaccine development is framed by two competing observations: 1) live attenuated SIV vaccine can be fully protective *in vivo* (3–6); and 2) uncontrolled replication of a live attenuated retrovirus has a finite risk for emergence of virulence as a result of genetic errors produced over time by reverse-transcriptase (11, 12). Within this context, a live attenuated HIV-1 whose replication is not constitutive but is instead conditionally regulated (such that rounds of reverse transcription with accompanying potential for error are strictly limited) might yield a paradigm that minimizes evolution to virulence. Previously, we and others (13, 14) have broached the concept of conditional control of HIV-1 and SIV through gain-of-function. Here, we describe the design for a constitutively dead HIV-1, which can be conditionally resuscitated to live by tetracycline.

MATERIALS AND METHODS

Vectors—pHIV-DoxT and pHIV-DoxSp were constructed in parallel. Initially, both LTR changes were generated in a subgenomic shuttle vector, p-43CAT, which contained pNL4-3 sequences from nucleotides −43 through +110 positioned upstream of a CAT reporter gene (15). First, TAR function was inactivated by mutation using PCR as described previously (16). Next, a 302-bp DNA fragment, which contains

[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; SIV, simian immunodeficiency virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair(s); RTTA, reverse tetracycline-controlled *trans*-activator; ELISA, enzyme-linked immunosorbent assay; Dox, doxycycline; CTL, cytotoxic T-lymphocyte.

seven, 19-bp inverted repeat sequence of the operator O_2 of Tn*10* was generated from pUHC13-3 (generously provided by Gossen and Bujard (17)). This *tet*-operator fragment was ligated into p-43CAT at a position upstream of the HIV TATAA box to create a reporter plasmid named TetopTCAT. To add three Sp1 sites to TetopTCAT, an oligonucleotide (GGGAGGCGTGGCCTGGGCGGACTGGGGAGTGGCG) was inserted 21 bases upstream of the TATAA box. This Sp1 site containing plasmid was named TetopSpCAT. The modified HIV-1 promoters from TetopTCAT and TetopSpCAT were then separately swapped into the 3′ LTR of full-length pNL4-3 molecular genome. Subsequently, the proviral genomes were further modified by oligonucleotide-based cloning such that the first 40 nucleotides of the 3′ U3 region were positioned immediately downstream of the polypurine tract and upstream of the *tet*-operator (tetO) sequences. A cDNA for the reverse tetracycline-controlled activator (RTTA) was amplified from p172-Neo (18) and substituted in place of Nef-coding sequence immediately downstream of the *nef*-AUG. Finally, the TetopSp1 promoter and TAR were also transferred into the 5′ LTRs of full-length molecular genomes to proviruses HIVDoxT and HIVDoxSp. Genotypically, HIVDoxT is *tat*(+)*tar*(−)*nef*(−)Sp1(−) and HIVDoxSp is *tat*(+)*tar*(−)*nef*(−)Sp1(+).

Cells and Transfections—293T cells, maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, were used for transfections. Plasmids were transfected into subconfluent cells with LipofectAMINE-plus according to the manufacturer's protocol (Life Technologies, Inc.). Where used, doxycycline (Sigma) was added to cells at a final concentration of 2 μg/ml.

CAT Assays—Assays for chloramphenicol acetyltransferase activity were performed in either of two ways. One, the ^{14}C-labeled chloramphenicol was used in a standard enzymatic based TLC assay, as described previously (15). Two, a commercial kit (Roche Molecular Biochemicals) was used according to the manufacturer's protocol. This kit measures total protein concentration in an ELISA format.

p24 ELISA—HIV-1 capsid, p24, ELISA was performed according to the manufacturer's protocol (Zeptometrix, Buffalo, NY). In brief, various dilutions of supernatant were incubated in wells coated with anti-p24 antibody. After six washes, biotin-labeled anti-p24 antibody was added and incubated for 1 h. After washing, streptavidin peroxidase was added for 30 min. The wells were washed and peroxidase substrate was added. Color development was terminated after 30 min by the addition of stop solution. The optical density of each well was determined at 450 nm in a plate reader.

Magi Cell Assay—Magi cells were obtained from the NIH AIDS Research and Reference Reagent Program and were contributed by Michael Emerman (19). Assays were performed according to the recommended protocol. Cells were exposed to virus for at least 6 h. Two days post-infection, the cells were fixed for 5 min with 0.2% glutaraldehyde, 1% formaldehyde in phosphate-buffered saline. Cells were washed twice with phosphate-buffered saline and then staining solution (4 mM ferrocyanide, 4 mM ferricyanide, 2 mM $MgCl_2$, 0.4 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)) was added and incubated at 37 °C for 1 h. Blue cells were counted.

Quantitative RT-PCR—Viral stocks were normalized for p24 content. RNA was extracted using the Qiagen Viral Amp kit and then treated with DNase I (1 unit/10 μl at 37 °C for 1 h). DNase I was removed with Ambion's DNA-*free*™. Samples were then analyzed by Roche's HIV Amplicor™ assay. Final viral genomic copy numbers were determined based on an internal control.

PCR—Cellular DNA was extracted using standard proteinase K techniques. 200 ng of cellular DNA was used per PCR reaction, which was performed with *Taq* polymerase-containing SuperMix™ (Life Technologies, Inc.) and 200 nM concentration of each HIV specific primer (*pol3*, ggttggtcagtgctggaatc; *pol4*, cctgtctctgctggaattac). Reactions were cycled 45 times at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

RESULTS

Tetracycline-regulated HIV-1 Promoters—Transcription from the HIV-1 provirus is dependent on its 5′ LTR, which contains three Sp1 motifs (15), and on virally encoded transactivator Tat protein (reviewed in Ref. 20). Expression from the LTR is controlled by Tat in a manner specified by a TAR RNA sequence (21) present in the R-region of the LTR. The intact Tat/TAR axis is absolutely required for HIV-1 viability (reviewed in Ref. 20); loss of either component leads to replication incompetence. Based on this strict requirement, we sought to create a constitutively "dead" HIV-1 genome by engineering an

inactivating mutation into TAR (16). This TAR(−) context would then permit us to ask whether an unrelated tetracycline-regulatable transcription mechanism could conditionally resuscitate an otherwise dead HIV-1 to live.

We addressed this hypothesis stepwise. First we queried whether the HIV-1 LTR could be converted from Tat-dependent to tetracycline-dependent transcription. Starting with a minimal LTR reporter plasmid (p-43CAT; Ref. 15), which contained as a promoter only the HIV-1 TATAA box and downstream TAR sequence, we removed completely Tat responsiveness by mutating TAR nucleotides +24 to +32, changing TGAGC-CTGG to CCTCGGACC (16). Seven *tet*-operators were then inserted upstream of TATAA to create the TetopTCAT reporter plasmid (Fig. 1). Because the authentic HIV-1 TATAA box in the viral LTR is 5′ flanked by three Sp 1 sites, which are important for function (22), we next added synthetically three such sites to TetopTCAT creating a second reporter plasmid, TetopSpCAT (Fig. 1).

Previously, *tet*-operator-ontaining promoters have been shown to be inducible by tetracycline in the context of a reverse tetracycline-controlled *trans*-activator, RTTA (developed by Gossen and Bujard; Refs. 17 and 18). We, thus, tested the responsiveness of TetopTCAT and TetopSpCAT to RTTA/Dox in transient co-transfections. Consistent with their TAR mutation, neither TetopTCAT or TetopSpCAT responded transcriptionally to Tat (Fig. 1, *B* and *C*). However, expression of both reporters were induced by a RTTA plasmid. We noted some leakiness of RTTA phenotype in these plasmid co-transfections in that some activity was observed even in the absence of Dox (a tetracycline derivative) (Fig. 1*C*). However, enhanced activation by RTTA was clearly documented for both TetopTCAT and TetopSpCAT in the presence of doxycycline. In comparing TetopTCAT and TetopSpCAT, a slightly superior relative induction profile was seen for the former rather than the latter.

Doxycycline-regulated Expression of Recombinant HIV-1 Proviruses—Because basal LTR behavior and threshold of LTR responsiveness to activators can be quite different between transfected subgenomic LTR reporters *versus* replicating proviral genomes (23), we next verified doxycycline regulation in proviral contexts. Full-length HIV-1 pNL4-3 genomes containing either TetopT- or TetopSp-formatted LTRs were constructed. Because Nef is dispensable for HIV-1 replication in many cell culture systems (24, 25), to create an autonomous RTTA/dox axis, we inserted into *nef* of a proviral genome an RTTA cDNA. RTTA was positioned in frame to the authentic Nef-AUG. The insertion of RTTA was performed in a manner that removed Nef-coding sequences and resulted in loss of Nef function. Two thus generated RTTA-expressing genomes were named pHIV-DoxT and pHIV-DoxSp. Fig. 2*A* illustrates schematically these proviruses, which differ only in their LTRs. Both pHIV-DoxT and pHIV-DoxSp genomes were sequenced in their entirety. Sequencing results confirmed that the proviruses were indeed as designed with no significant adventitious changes fortuitously introduced during the construction process (data not shown). Integrated pHIV-DoxT and pHIV-DoxSp proviruses are expected to have the respective modified Tet-reponsive promoters in 5′ and 3′ LTRs.

pHIV-DoxT and pHIV-DoxSp were checked for regulation by tetracycline. Both genomes are genetically *tar*(−) and would ordinarily be expected to be wholly defective in producing viral proteins and/or particles. However, following transfection into 293T or HeLa cells, both proviruses, in a doxycycline-dependent fashion, capably released Gag (as measured by a CA-p24 ELISA) and RT (as measured by enzymatic RT assay) from cells. In the absence of doxycycline, no replication competent virus could be recovered. Fig. 2*B* shows that levels of Gag and
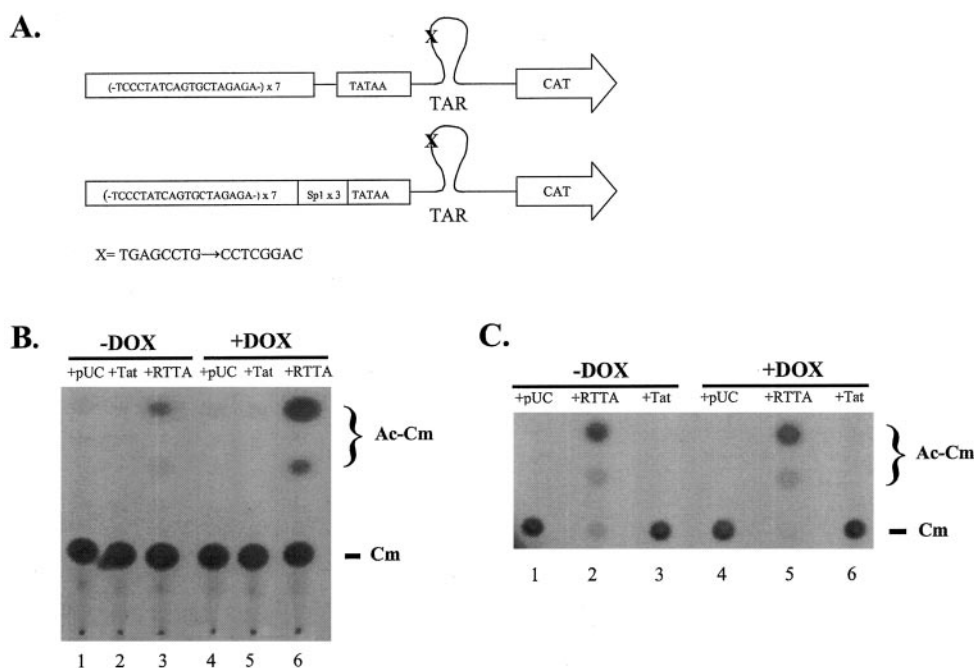
FIG. 1. **Construction of tetracycline-responsive promoters.** *A*, schematic illustration of tetopT (*upper*) and tetopSp (*lower*) promoters. In tetopT a 19-bp *tet* O sequence is repeated seven times in the insertion into the HIV-1 minimal TATAA promoter. TetopSp has three Sp1 sites inserted between the *tet* O sequences and the TATAA box. TAR is mutated in both promoters, as shown. *B*, transactivation of tetopT with RTTA. TetopTCAT was co-transfected into HeLa cells with either pUC (*lanes 1* and *4*), a Tat expression vector (*lanes 2* and *5*), or an RTTA-expression vector (*lanes 3* and *6*) in duplicate wells. Doxycycline (final concentration = 2 μg/ml) was added at 24 h to one well (*lanes 4–6*) of each set of co-transfections. At 48 h, CAT assay on protein extracts from each transfection was performed. Tat had no effect on CAT production. RTTA co-transfection resulted in acetylation of 6% of the chloramphenicol in the absence of doxycycline. This level of activation increased to 38% (7.7-fold) with doxycycline induction. *C*, transactivation of tetopSp with RTTA. TetopSpCat was co-transfected with pUC (*lanes 1* and *4*), an RTTA expression vector (*lanes 2* and *4*), and a Tat-expression vector (*lanes 3* and *6*). One of each set of wells was treated with doxycycline (*lanes 4–6*). Tat and the pUC control had no effect on CAT acetylation. Repeat CAT assay of the RTTA samples in the linear range of acetylation shows Dox induction to be also ~7-fold (data not shown).

RT produced by transient transfection were induced in excess of 25-fold by doxycycline. These findings suggest that the heterologous RTTA + Dox mechanism substituted effectively for the Tat/TAR axis.

Proviral-based doxycycline regulation of HIV-1 expression was further characterized by Western blotting. We transfected pHIV-DoxT, pHIV-DoxSp, and the control molecular clone pNL4-3 (wild-type HIV-1) into 293T cells in the presence of doxycycline. 48 h later, all transfected, Dox-treated cells expressed HIV-1 p24 as verified by ELISA of the culture supernatants. The Dox-inducible proviruses produced slightly lower amounts of p24 compared with pNL4-3 (Fig. 2*B*). Total cell lysates were also examined by Western blotting using either hyperimmune HIV immunoglobulin (HIVIG) or monoclonal anti-gp160 serum (Fig. 3, *A* and *B*), and we observed that HIV-DoxT and HIV-DoxSp produced HIV-1-specific Env and Gag profiles qualitatively indistinguishable from that of NL4-3.

A further test for proper expression from HIV-DoxT and HIV-DoxSp is to test for the intact biological function of a viral gene product. We, next, compared Tat function from HIV-Dox viruses to that produced from wild type pNL4-3. Although the *cis*-TAR elements in pHIV-DoxT and pHIV-DoxSp were disabled by mutation, synthesis of Tat from these genomes could be measured in *trans* using an HIV-1 LTR CAT reporter. Hence, we co-transfected an HIV-1 LTR CAT plasmid into 293T cells separately with pNL4-3 or pHIV-DoxSp or pHIV-DoxT in the presence of doxycycline (2 μg/ml). All three proviral genomes were found to activate Tat-dependent CAT expression comparably (Fig. 3*C*), suggesting (within the limits of this assay) equivalence in functional Tat synthesis. These results agree with the Western blot findings and support that RTTA/Dox activation of a *tet*-operator-containing LTR (*i.e.* pHIV-DoxT; pHIV-DoxSp) largely recapitulates that normally provided by Tat activation of

TAR in a wild-type HIV (*i.e.* pNL4-3) LTR.

HIV-Dox Genomes Assemble Morphologically Intact Virions—To ask whether RTTA/Dox expression from HIV-DoxT and HIV-DoxSp produces morphologically correct virions, the maturation and release of viral particles were analyzed by electron microscopy. 293T cells were individually transfected with pNL4-3, pHIV-DoxT, or pHIV-DoxSp and treated with doxycycline (2 μg/ml). Cells, productive for viral proteins as verified by p24 ELISA, were then examined by electron microscopy. Both pHIV-DoxT and pHIV-DoxSp (Fig. 4, *top* and *bottom*) were seen to produce and release mature viral particles at a frequency indistinguishable from wild-type HIV-1, pNL4-3 (data not shown).

pNL4-3, HIV-DoxT, and HIV-DoxSp virions were further analyzed for packaging of genomic viral RNA. Individual viral stocks, prepared from transfections of proviral plasmids into 293T cells, were normalized for p24 and pelleted. Following protein extraction, virion-associated RNAs were treated with DNase I and analyzed by quantitative RT-PCR. pNL4-3, HIV-DoxT, and HIV-DoxSp had 1.5×10^5, 0.75×10^5, and 1.2×10^5 copies/ng of p24, respectively. This assay has a 95% confidence index of 0.5 log. We therefore concluded that HIV-Dox virions packaged viral RNAs with efficiencies similar to that of wild-type HIV-1 virions.

A Highly Attenuated Replication Phenotype for HIV-DoxT and HIV-DoxSp—We next tested HIVDoxT and HIVDoxSp for productive replication in T-cells. Surprisingly, we found that even in the presence of doxycycline, neither virus was highly lytic nor highly productive of supernatant RT or p24 over 6 months in tissue culture (data not shown). However, both genomes did replicate to a low level in a Dox-dependent manner as assayed by PCR identification of integrated proviral DNA (Fig. 5). Thus HIVDoxT and HIVDoxSp have a highly attenu-

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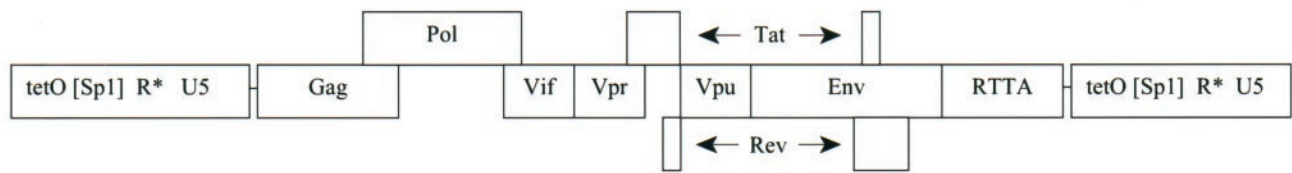
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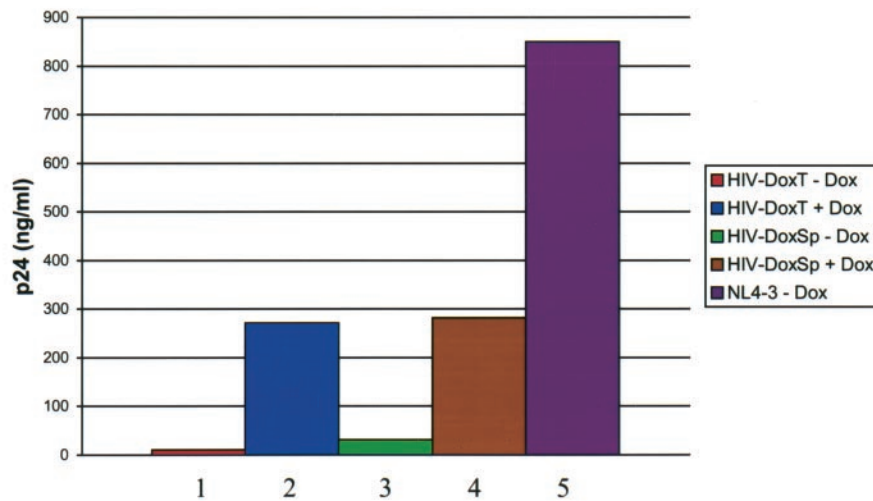
FIG. 2. **Construction of proviral genomes regulated by tetracycline.** *A*, HIV-Dox genomes. LTRs were altered as described under "Materials and Methods". The gene for RTTA was inserted in frame into *nef*. All other viral reading frames were entirely unchanged. pHIV-DoxT and pHIV-DoxSp differ only in the LTRs, where pHIV-DoxSp has three Sp1 sites and pHIV-DoxT has none. *B*, proviral gene expression is induced by doxycycline. pHIV-DoxT, pHIV-DoxSp, or pNL4-3 was individually transfected into 293T cells in six wells on day 0. The cells were washed with media on day 1 and doxycycline (final concentration = 2 μg/ml) was added to three of the six wells. On day 3, the p24 level in each supernatant was determined. The p24 values (averages of three determinations) are shown.
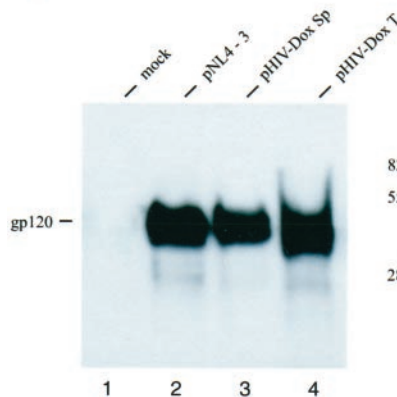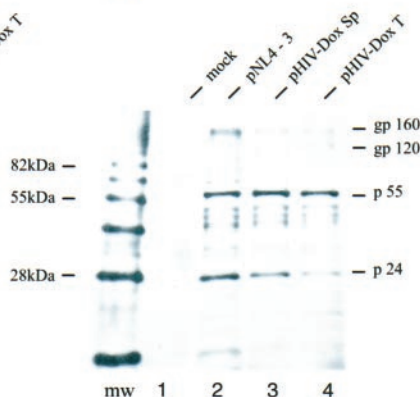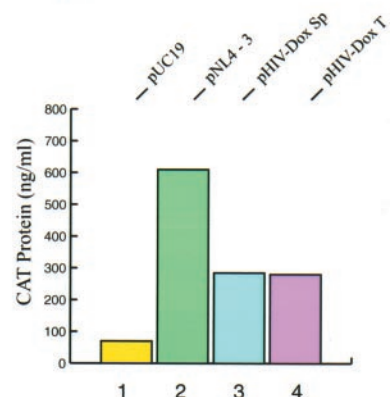
FIG. 3. **Intact expression of HIV-1 proteins from CDCL genomes.** HIV-Dox genomes express wild-type Env and Gag proteins. Western blot analysis with an anti-gp120 antibody (*A*) or with a polyclonal hyperimmune HIV-1 serum (*B*) was performed on cell lysates after transfection with no plasmid (*lane 1*), pNL4-3 (*lane 2*), pHIV-DoxSp (*lane 3*), and pHIV-DoxT (*lane 4*). (*mw*, molecular mass markers). *C*, HIV-Dox genomes synthesize intact functional Tat, which transactivates an HIV-1 LTR-CAT reporter. HIV-1 LTR CAT reporter was co-transfected into 293T cells alone (*lane 1*), with pNL4-3 (*lane 2*), pHIV-DoxSp (*lane 3*), or pHIV-DoxT (*lane 4*). Doxycycline (2 μg/ml) was added to each well on day 0 of transfection. Protein extracts were prepared on day 3 after transfection and analyzed by CAT ELISA.

ated replication phenotype, which is somewhat incongruous with their capacity to express Dox-dependent viral protein synthesis to levels indistinguishable from that of pNL4-3 (Figs. 2 and 3).

The serial samplings (Fig. 5) of tissue culture-propagated HIVDoxSp were analyzed to verify for intactness of the engineered mutation in TAR. Fig. 6 shows that the nine-nucleotide substitution in the loop of TAR previously shown to be wholly inactivating for HIV-1 replication (16) was entirely unchanged at days 85 and 89. In further assays, we repeatedly noted that our engineered TAR mutation resulted in reduced reverse transcription of the HIV-DoxT and HIV-DoxSp genomes (data not shown). This observation agrees with emerging findings that Tat/TAR plays a role in reverse transcription of HIV-1 (26–28).
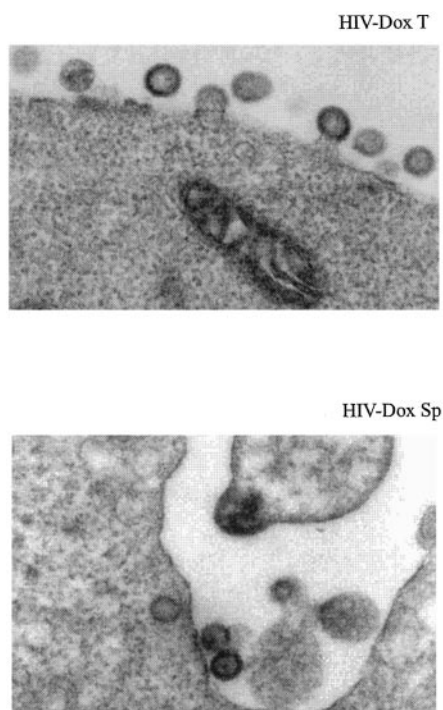
FIG. 4. **CDCL genomes produce morphologically intact viral particles.** pHIV-DoxT (*top*) and pHIV-DoxSp (*bottom*) were transfected into 293T cells. On day 2 after transfection the cells were fixed with glutaraldehyde. Electron microscopy was performed. The *upper panel* shows HIV-DoxT virions, and the *lower panel* shows HIV-DoxSp virions.

In this regard, the robust viral expression profiles (Figs. 2 and 3) of HIVDoxT and HIVDoxSp coupled with their highly attenuated replication phenotype may describe an optimal combination for an HIV-1 vaccine. Efficient intracellular synthesis of HIV-1 antigens, in principle, would serve to elicit major histocompatibility class I-driven cell-mediated immunity against virus-infected cells (reviewed in Ref. 29), while an attenuated regulatable replication phenotype would serve to minimize reverse-transcription errors, which could fuel reversion of innocuous vaccine entities to virulent counterparts. We (P. Marx) are currently evaluating the validity of this reasoning through the *in vivo* inoculation of pHIV-DoxT and pHIV-DoxSp into nonhuman primates.

DISCUSSION

Vaccines have been one of the most important medical achievements for infectious diseases. Over the past century, successful implementation of vaccines has led to the control of smallpox, measles, rubella, tetanus, and diphtheria among many other pathogens. In the face of the modern HIV/AIDS pandemic in which 15,000 new infections occur each day, 21.8 million individuals cumulatively have succumbed to AIDS, 13 million children have been orphaned by this scourge, and 34 million people are currently infected with HIV, it is glaringly regrettable that no effective and safe AIDS vaccine is yet within sight. Indeed, it is sobering that after 15 years of research and development only one vaccine concept, a recombinant gp120 subunit protein, has reached phase III clinical trial. Even more disappointing is the fact that the gp120 subunit approach, at best, solely elicits antibody immunity to HIV. The natural course of HIV-1 infection *in vivo* suggests that the virus can effectively evade humoral immunity, and few vaccinologists currently believe that a vaccine, which induces only antibodies without a cytotoxic T-lymphocyte (CTL) response, has any real chance for success against HIV-1 (30).

All current vaccines fall within two broad concepts, live or dead. Live vaccines encompass attenuated natural microbes (viruses, bacteria, etc.) as well as recombinant vectors, which provide immunogenicity at reduced pathogenicity (reviewed in Ref. 31). Dead vaccines include inactivated whole pathogens and protein subunits of pathogens. Both live and dead vaccines elicit antibody responses. However, only live vaccines can utilize efficiently the intracellular major histocompatibility class I pathway to induce CTL. Dead vaccines, on the other hand, are generally safe with no potential for reversion from avirulence to virulence. Considering the attributes of both live and dead vaccines, one might reason that a dead/live hybrid HIV vaccine could offer adequate immunogenicity with an acceptable safety profile.

No vaccine is entirely risk-free. Extant data from SIV vaccine research support the notion that a live attenuated virus can be sufficiently protective *in vivo* against disease development, albeit currently with an unacceptable risk of reversion to virulence (6–8). With the aim of a safer live attenuated virus, we have constructed two forms of HIV-1 (pHIV-DoxT and pHIV-DoxSp) that marry the dead + live concepts of vaccine design. Thus pHIV-DoxT and pHIV-DoxSp are constitutively dead by virtue of being TAR(−). Each can, however, be conditionally resuscitated to live for expression of proteins, replication of genome, and assembly of mature viral particles. Indeed, upon provision of doxycycline, both genomes compare favorably to wild-type HIV-1 in robustness of expression, synthesizing all viral proteins except Nef (Fig. 3, *A* and *B*). The Nef deletion in these genomes provides an additional safety feature, since various *in vivo* findings have verified *nef(−)* HIV-1 to be highly attenuated in pathogenicity when compared with its wild-type counterpart (9, 10, 32, 33). Thus it is our persuasion, supported in part by experimental findings (Fig. 5), that pHIV-DoxT and pHIV-DoxSp have highly attenuated replication such that while these viruses can infect cells, actively synthesize viral antigens, and replicate *in vivo*, they do so with an efficiency unlikely to cause disease.

A recent International AIDS Vaccine Initiative report (34) criticizes the absence of better vaccine designs as a basic research barrier to successful vaccine development. International AIDS Vaccine Initiative recommended that "it is imperative that new and innovative vaccine designs capable of eliciting the broadest and most robust immune responses against HIV be considered" (35). How might HIV-Dox fulfill this recommendation? We note that all reading frames (except Nef) are intact in HIV-Dox, and viral protein expression in the presence of doxycycline is to wild-type levels. Currently, it is not known which HIV proteins are the best immunogens. While most vectors have been designed to express structural proteins, such as Gag and Env, new animal studies indicate that accessory proteins, such as Tat, may elicit more protective immune responses (36). Consequently, a vector such as HIV-Dox, which produces the full complement of HIV-1 proteins except Nef, offers perhaps the best opportunity for broad immunity. Furthermore, the conditionally live aspect of HIV-Dox provides for CTL induction, an option not effectively achievable with protein subunit inoculation.

Perhaps the most significant concept introduced by the CDCL genomes is that the constitutive ability of live attenuated viruses to autonomously evolve is now subject to interruption. It is well established that the propensity of attenuated precursor viruses to evolve to virulent progenies correlates directly with the number of replicative opportunities the former is afforded *in vivo* (37, 38). Some rounds of intracellular replication of HIV-1 are clearly required for CTL elicitation; however, beyond that, each additional round presents incremental opportunity for acquisition of genome changes with attendant risks of increased virulence. Conventional live atten-
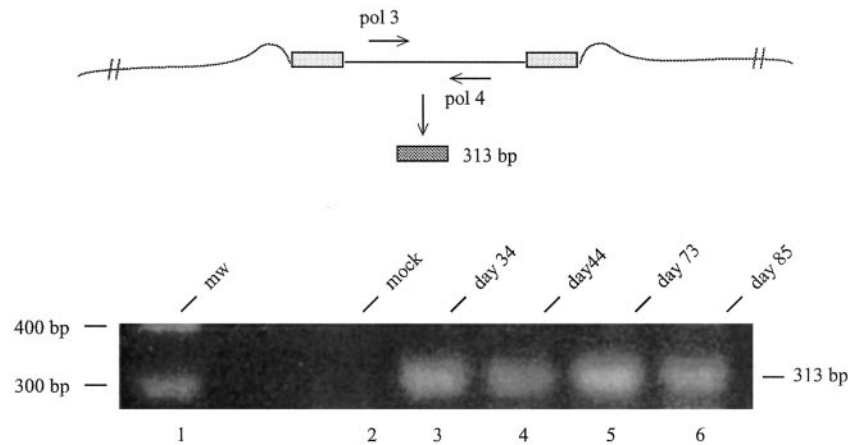
FIG. 5. **Attenuated replication of CDCL virus in Jurkat T-cells.** *Top*, schematic representation of PCR amplification of integrated provirus from genomic cell DNA using *pol3* and *pol4* primers. PCR-amplified band is expected to be 313 bp in size. *Bottom*, PCR amplification of DNA from Jurkat cells infected with HIV-DoxSp. 5×10^6 cells were infected with HIV-DoxSp (200 ng of p24 input) on day 0. At the indicated times, total cell DNA was extracted and analyzed by PCR. The products were visualized on ethidium bromide-stained agarose gel (*lane 1*, molecular size standards; *lane 2*, uninfected cells; *lane 3*, day 34 cells; *lane 4*, day 44 cells; *lane 5*, day 73 cells; and *lane 6*, day 85 cells). We note that at these various time points no p24 values above 12.5 pg/ml were detected in culture supernatants. Similar results were observed for HIV-DoxT (data not shown).
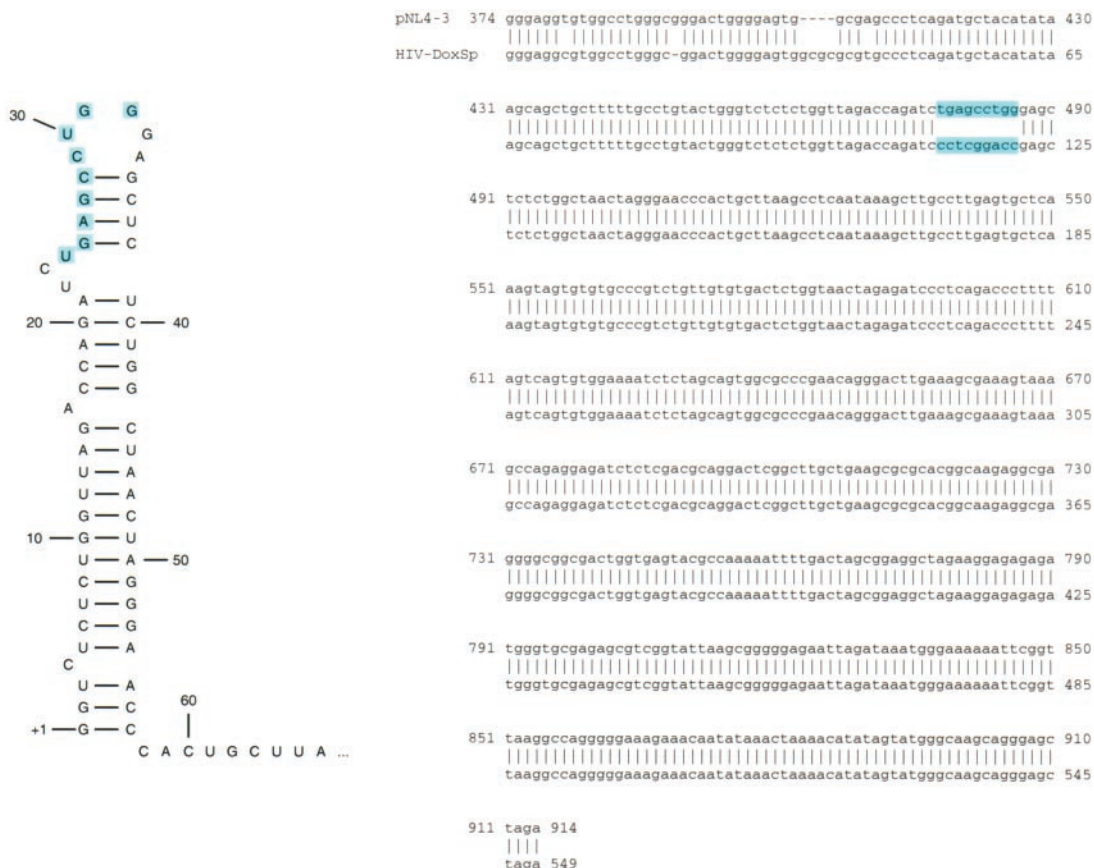

FIG. 6. **Stability of TAR mutation in HIV-DoxSp after prolonged propagation in Jurkat cells.** Jurkat cells were infected on day 0 with HIV-DoxSp viral stock and washed on day 1. The cells (same culture as shown in Fig. 5) were serially sampled over 89 days of culturing. Proviral DNA was extracted and PCR amplified with forward primer 5′-TCGAGCTATCGATCATTACG-3′ (located in the U3 region of HIV-DoxSp) and reverse primer 5′-GGCCAGGATTAACTGCGAATCGT-3′ (which corresponds to positions 937–915 of the parental NL4-3 provirus). The amplified products from days 85 and 89 were cloned. Three independent clones from day 85 and three independent clones from day 89 were directly sequenced in their entirety (549 nucleotides). All six clones had the identical sequence, which was the same as that in the original HIV-DoxSp input virus used on day 0. An alignment of this conserved HIV-DoxSp sequence with parental NL4-3 is shown on the *right*. Notably, the nine-nucleotide mutation (highlighted in *blue*) engineered into the loop of wild-type TAR RNA (*left*), previously shown to fully inactivate the Tat/TAR-axis (16), was uniformly maintained with no evidence of reversion. Other mismatches between HIV-DoxSp and NL4-3 sequences were present from the construction of the original HIV-DoxSp genome and were not changes that occurred during propagation of the infection.

uated HIV-1 approach offers no control against this process of virulent evolution. By contrast the CDCL approach mandates a cessation to replication unless demanded by provision of doxycycline. Hence, unlike the conventional default, which drives replication, the CDCL default is entirely opposite. Thus CDCL can be superior to live in offering an otherwise unavailable possibility for fine-tuning replication only to the extent beneficial for CTL creation.

The CDCL genomes introduce a new safety feature yet considered in current live HIV vaccine designs. Presumably, this concept extends beyond tetracycline regulation to other yet anticipated means for producing constitutively dead conditionally live phenotypes. To the extent that live vaccines might ultimately be a viable solution for AIDS in developing (as well as developed) countries, we and others (39) suggest that CDCL vaccines represent an incremental refinement which merit discussion and further experimental investigation.

We suggest that the CDCL genomes provide improved safety profiles when compared with constitutively replicating live attenuated genomes. Our findings, which are independently supported by observations from others (39), indicate that the replication of HIV-DoxT and HIV-DoxSp are completely doxycycline-dependent. It is not fully understood as to what are the many determinants that govern kinetically the reversion of the various attenuating changes that have been experimentally engineered into replication competent HIV-1 proviruses. Empirically, some changes revert quickly (13, 40, 41), while others are durable (42). Currently, we have no evidence for (although we do not exclude a formal possibility of) the HIV-DoxSp or HIV-DoxT genome to revert to virulence during doxycycline-dependent replication. When uninterrupted replication was examined over 89 days (Fig. 6) to 6 months (data not shown), no changes were seen in our engineered mutation in TAR. Based on our experience, the 9-base pair mutation in TAR cannot be compensated by second site changes elsewhere in the HIV-1 genome. Hence, within that context, these *tar*(−) doxycycline-dependent proviruses remain fully attenuated.

Our current study suggests that in *ex vivo* tissue culture setting, the CDCL concept as embodied by the HIV-DoxT and HIV-DoxSp genomes presents a highly regulated and stably attenuated virus/cell model, consistent with that desired for an *in vivo* vaccine. We stress that proof-of-concept *ex vivo* does not imply *in vivo* applicability. Nevertheless, comprehensive and open discussion of such concepts is in keeping with the urgency for "new and innovative vaccine design" at the basic science level (IAVI recommendation; Refs. 34 and 35) and cannot but help spur *in vivo* vaccine refinements. Indeed, *in vivo* testing by us (P. Marx) of Dox-regulated viral genomes in macaques is underway.

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